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Androgen Receptor in Sertoli Cells is not Required for Testosterone-induced Suppression of Spermatogenesis, but Contributes to Sertoli Cell Organization in *Utp14b^{jsd}* Mice

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Abstract

Testosterone acting through the androgen receptor (AR) maintains the arrest of spermatogonial differentiation in juvenile spermatogonial depletion (*jsd* mutation in the *Utp14b* gene) mutant adult male mice. It is not known which of the somatic cell types expressing AR mediates this inhibition. To determine if Sertoli cells are responsible, we selectively eliminated AR in Sertoli cells in *jsd* mice containing a floxed-*Ar* gene and an anti-Müllerian hormone-*Cre* transgene. In these Sertoli AR-knockout (SCARKO)-*jsd* mice, spermatogonial differentiation did not recover. However, the normal organization of Sertoli cell nuclei was drastically disrupted in SCARKO-*jsd* mice, compared to SCARKO or *jsd* mice. In addition, the extent of ectoplasmic specializations was reduced, tight junctions were not found, vinculin, an anchoring protein found in ectoplasmic specializations, became uniformly distributed in the cytoplasm, and the adult Sertoli cells showed excess heterochromatin subjacent to their nuclear envelope. Despite the abnormalities in Sertoli cells in SCARKO-*jsd* mice, global suppression of testosterone action and levels was still effective in restoring the differentiated germ cells, and this was accompanied by an improved arrangement of Sertoli cell nuclei. We conclude that Sertoli cells are not targets for the testosterone-mediated inhibition of spermatogonial differentiation in *jsd* mice, and both AR in Sertoli cells and the presence of differentiated germ cells contribute to maintaining the organization of Sertoli cells within the seminiferous tubules.

Keywords

Testis; Spermatogonial differentiation; *jsd*; Azoospermia; AR; Vinculin

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Introduction

Juvenile spermatogonial depletion (*jsd*) mice are spontaneous mutants (Beamer et al, 1988) in the *Utp14b* gene (Rohozinski and Bishop, 2004; Bradley et al, 2004). While homozygous females are fertile, the adult male homozygous *jsd/jsd* ("*jsd*" is used to indicate the *Utp14b^{jsd}* mutation throughout the paper) mice (referred to as *jsd* mice) are sterile, which is their only phenotypic defect. In these males, there is a progressive loss of germ cells because the spermatogonial differentiation becomes arrested after the first several waves of spermatogenesis (Kojima et al, 1997). The only germ cells found in most seminiferous tubules of adult *jsd* mice are undifferentiated type A spermatogonia, which are able to proliferate, but instead of differentiating, they die through apoptosis (de Rooij et al, 1999). Spermatogonial transplantation experiments have shown that this arrest of spermatogonial differentiation is a results of intrinsic defects in spermatogonia (Boettger-Tong et al, 2000; Ohta et al, 2001) and gene expression studies showed that the *Utp14b* gene is almost exclusively expressed in germ cells, whereas a potentially compensating gene is more actively expressed in somatic cells (Zhao et al, 2007).

Previous studies (Matsumiya et al, 1999; Tohda et al, 2001) have demonstrated that suppression of testosterone by gonadotropin-releasing hormone (GnRH) analogs reverses the block of spermatogonial differentiation and consequently stimulates the recovery of spermatogenesis to the spermatocyte or early spermatid stage in *jsd* mice. Further studies (Shetty et al, 2001; Shetty et al, 2006) suggested that relatively high testosterone levels are required to maintain the arrest of spermatogonial differentiation in *jsd* mice and proved that the testosterone action is mediated by the androgen receptor (AR).

In the wild-type mouse testis, AR is localized to the somatic cells, including Sertoli, Leydig, peritubular myoid, and blood vessel smooth muscle, but is not expressed in germ cells (Zhou et al, 2002). The AR expression pattern in *jsd* mice has been shown to be the same as in wild-type testis (Meistrich et al, 2005). Thus, despite the fact that the primary defect in the *jsd* mice is in the germ cells, the action of testosterone in maintaining the block of spermatogonial differentiation in *jsd* mice would be mediated by AR in one or more types of somatic cells. The inhibition of spermatogonial differentiation by testosterone in irradiated rats (Kangasniemi et al, 1996) has also been shown to be due to damage to the somatic environment in the testis (Zhang et al, 2007). Recognition of the target cell responsible for the arrest of spermatogonial differentiation in *jsd* mice will be of importance in elucidation of the mechanism by which hormones mediate this arrest. In addition, the results may be applicable to reversal of toxicant-induced blocks in spermatogonial differentiation in rats, as well as for treatment of human oligospermia/azoospermia since transient suppression of intratesticular testosterone levels has been reported to facilitate sperm count recovery in human (Charny and Gordon, 1978).

Androgens and AR are absolutely essential for maintenance of spermatogenesis in normal testes. Global loss of functional AR leads to a testicular feminized phenotype (Lyon and Hawkes, 1970). In order to determine the relative roles of AR in different cell types in testis, it was necessary to develop cell-specific AR knockout mice. When *Ar* was ablated in Sertoli cells, germ cell development stopped at the spermatocyte (Chang et al, 2004; De Gendt et al, 2004) or early spermatid (Holdcraft and Braun, 2004) stages, suggesting that the AR in the Sertoli cells plays a critical role in maintaining the Sertoli cell's ability to support normal spermatogenesis. In contrast, deletion of *Ar* in peritubular cells had only small quantitative effects on spermatogenesis (Zhang et al, 2006) and germ cell *Ar* deletion had no effect on germ cell development (Tsai et al, 2006). Although the deletion of *Ar* in Leydig cells stopped germ cell development at the round spermatid stage (Xu et al, 2007), this effect of absence of AR in Leydig cells may only be a result of the low testosterone levels and not a result of absence of

other Leydig cell androgen-dependent factors. Thus, the Sertoli cell appears to be the primary testicular cell that mediates androgen support of normal spermatogenesis.

In order to determine whether AR in Sertoli cells is also the target for testosterone inhibition of spermatogonial differentiation in *jsd* mice, we eliminated AR selectively in Sertoli cells (Sertoli cell AR knock-out, SCARKO) in *jsd* mouse testis by using a *Cre-lox* conditional knockout strategy (De Gendt et al, 2004; Holdcraft and Braun, 2004). There was no recovery of spermatogenesis in *jsd* mice lacking AR in their Sertoli cells, demonstrating that the Sertoli cell is not the target cell that mediates the androgen-dependent block of spermatogonial differentiation. In addition, we noted several abnormalities in Sertoli cells of SCARKO-*jsd* mice, particularly in the positioning of their nuclei, which were much more dramatic than those observed in either SCARKO or *jsd* mice.

Methods

Animals

Transgenic mice with floxed-*Ar* (*Ar^{flox}*) on a 129/Sv genetic background were obtained from the Catholic University of Leuven, Belgium (De Gendt et al, 2004). Both males and females were crossed with mice carrying the *jsd* mutation on a C3H-B6-129 (HB129) mixed background (Bolden-Tiller et al, 2007), and the offspring were intercrossed for several generations to generate *Ar^{flox}* and *jsd* homozygous double-mutant females (*Ar^{flox/flox}*, *Utp14b^{jsd/jsd}*). C57BL/6 mice expressing *Cre* recombinase under the control of the anti-Mullerian hormone (*Amh*) promoter (*Amh-Cre* mice, *Cre^{+/-}*) were obtained from the University of Washington (Holdcraft and Braun, 2004). Both males and females (*Cre^{+/-}*, *Utp14b^{+/+}*) were also crossed with mice carrying the *jsd* mutation for one or more generations to generate double-mutant males that were heterozygous for both *Amh-Cre* and *jsd* (*Cre^{+/-}*, *Utp14b^{+/-jsd}*). The *Ar^{flox/flox}*, *Utp14b^{jsd/jsd}* females were then mated with *Cre^{+/-}*, *Utp14b^{jsd/+}* males to produce the triple-mutant test model males (*Ar^{flox/Y}*, *Cre^{+/-}*, *Utp14b^{jsd/jsd}*, SCARKO-*jsd*). Since *Amh* is specifically expressed in the Sertoli cells in the testis, the *Cre* recombinase is expected to be expressed only in Sertoli cells and recombine the floxed sites in the *Ar* gene, leading to inactivation of the *Ar* gene selectively in Sertoli cells (Holdcraft and Braun, 2004). The wild-type, *jsd*, and SCARKO controls were chosen from littermates of the test models. The wild-type and *jsd* controls carried *Ar^{flox}*, and some of the wild-type and SCARKO controls were *jsd* heterozygotes. PCR genotyping of tail DNA was used to identify mice with *Ar^{flox}* (De Gendt et al, 2004), *Amh-Cre* transgenes (Primers: forward: 5'-tggtttcccgagacacctaag-3', reverse: 5'-gagcctgtttgcacgttcacc-3'), and *Utp14b^{jsd}* mutants (Shetty et al, 2001). All animal studies were approved by the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Hormone and Hormone Antagonist Treatment

The hormone and hormone analogues were given as previously described (Shetty et al, 2006). Briefly, GnRH antagonist, acyline (obtained from the Contraceptive Development Branch of NICHD, North Bethesda, MD), was subcutaneously injected twice, at doses of 20 and 10 mg/kg-body weight at 8 and 10 weeks of age, respectively, and the mice were killed at 12 weeks of age. For treatment with flutamide (an AR antagonist) or testosterone, two 2-cm Silastic capsules (Dow Corning, Midland, MI), filled with flutamide (Sigma, St. Louis, MO), or one 2-cm capsule filled with testosterone (Sigma, St. Louis, MO) were implanted subcutaneously at 8 weeks of age and left in place until the mice were killed 4 weeks later.

Testicular Histology

After the mice were killed, testes were removed and fixed in Bouin's solution and embedded in methacrylate. The blocks were sectioned and stained either with hematoxylin only or hematoxylin and periodic acid Schiff's (PAS) reagent.

The recovery of spermatogenesis was evaluated by the tubule differentiation index (TDI) as previously described (Shetty et al, 2001). The TDI is defined as the percentage of tubules that contain three or more differentiating germ cells at the B spermatogonial stage or beyond.

For scoring the abnormal Sertoli cell nuclear organization, the tubules were placed into one of four categories: Tubules with all the Sertoli cell nuclei aligned along the basement membrane (normal tubules); tubules with a few (less than 5) Sertoli cell nuclei displaced from the basement membrane; tubules with 5 or more randomly positioned Sertoli cell nuclei displaced from the basement membrane; tubules with a cluster containing at least 5 (usually degenerating) Sertoli cell nuclei.

Immunostaining and confocal microscopy

Testes were removed and fixed in 4% paraformaldehyde for up to 24 hours. The fixed tissues were embedded in paraffin, and sectioned, and the sections were deparaffinized and subjected to antigen retrieval.

Sections were sequentially incubated with AR antibody (PG21, (Prins et al, 1991)) at a 1:200 dilution overnight at 4°C, biotinylated anti-rabbit IgG, and then with an avidin-biotin-peroxidase complex reagent (Vectastain *Elite* kit, Vector Laboratories, Burlingame, CA). The staining was developed by incubation with the peroxidase substrate diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin.

For immunofluorescent staining of vinculin and vimentin, the sections were incubated with monoclonal, mouse anti-human antibodies either to vimentin (1:200 dilution) or to vinculin (1:300 dilution) (Sigma, St. Louis, MO) overnight at 4°C. After washing in PBS, the slides were then incubated with Alexa Fluor 594-conjugated goat anti-mouse antiserum (IgM, 1:200, Molecular Probes, Carlsbad, CA) for vimentin or Alexa Fluor 488-conjugated goat anti-mouse (IgG, 1:200, Molecular Probes, Carlsbad, CA) for vinculin. The testis sections were examined using a confocal microscopy system (LSM 510, Carl Zeiss, Oberkochen, Germany).

Real-time RT-PCR

Testis samples were collected from *jsd* and SCARKO-*jsd* mice (n = 4 for each group) and total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA), with DNase I treatment to digest genomic DNA. The cDNA was generated with a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences, Indianapolis, IN). Quantitative real-time PCR of the cDNA was performed using the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) and SYBR Green (JumpStart Taq Ready Mix, Sigma, St. Louis, MO) detection to determine the differential expression of the *Rhox5* gene. The housekeeping gene, beta-actin, was used to normalize concentration values for each sample. The following primers were used: *Rhox5*: forward 5'- ggcccaagctcagaatc -3', reverse 5'- ctgaataggatcaatgatgaag -3'; beta actin: forward 5'- tgacaggatgcagaaggagat -3', reverse 5'- tactcctgcttgctgatccac -3'. All samples were run in triplicate.

Electron Microscopy

Tissues were prepared as described previously (Chiarini-Garcia and Russell, 2001). Briefly, whole-body perfusion of mice was performed through the right atrium with 5% glutaraldehyde in 0.05 M cacodylate buffer for 15-20 minutes. Collected testes were fixed in glutaraldehyde

overnight, and then stored in cacodylate buffer. After overnight postfixation with 1% buffered osmium tetroxide and 1.25% potassium ferrocyanide, samples were dehydrated and embedded in Araldite 502 medium. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

Statistics

All quantitative data were represented as arithmetic means $\pm$ SEM. ANOVA and subsequent Student-Newman-Keuls post-hoc analysis for pairwise comparisons (Table 1, Table 2), and independent-samples *t*-tests (Figure 3) were performed to determine the significance of differences ($P < 0.05$) between groups using the SPSS v 12.0 statistical software package (SPSS, Inc, Chicago, IL).

Results

Characteristics of *jsd* mice with Ar deletion in Sertoli cells

The body weights of wild-type, SCARKO, *jsd*, and SCARKO-*jsd* mice were comparable (data not shown). The seminal vesicle weights were not significantly different among wild-type, SCARKO, *jsd*, and SCARKO-*jsd* mice (Table 1), indicating that circulating androgen levels were similar. The testis weights of SCARKO and *jsd* mice were both markedly reduced to 26% and 18%, respectively, of that of wild-type mice (Table 1). The testis weights of SCARKO-*jsd* mice were even lower than both those of *jsd* ($P = 0.054$) and of SCARKO ($P < 0.05$) mice.

To determine whether AR was selectively ablated in Sertoli cells in SCARKO-*jsd* mice, we examined testicular AR expression by immunohistochemical staining and compared it with that in wild-type, *jsd*, and SCARKO littermates. In wild-type and *jsd* mice (Figure 1 A, C), AR was detected in Sertoli cells, myoid peritubular cells, vascular smooth muscle cells, and Leydig cells. In SCARKO and SCARKO-*jsd* mice (Figure 1 B, D), while AR staining was still positive in the other testicular somatic cells, AR expression was eliminated in 99.5% of Sertoli cells of SCARKO-*jsd* mice.

To confirm that AR function was absent in the Sertoli cells of SCARKO-*jsd* mice, the mRNA levels of *Rhox5*, a Sertoli cell-specific gene that is regulated by AR (Rao et al, 2003), were measured by real-time RT-PCR. The level of *Rhox5* transcripts normalized to beta-actin in SCARKO-*jsd* testes was 0.034 ± 0.016 times the level of that in *jsd* testes, indicating a successful knock-out of AR function in the Sertoli cells.

Spermatogonial Differentiation in SCARKO-*jsd* Mice

If AR in Sertoli cells mediated the effect of testosterone on inhibition of spermatogonial differentiation in *jsd* mice, selective ablation of AR in Sertoli cells of *jsd* mice should reverse this inhibition. On other hand, if AR in Sertoli cells is not the mediator, spermatogonial differentiation should be blocked in SCARKO-*jsd* mice to the same extent as in *jsd* mice. The testes of SCARKO-*jsd* mice (Figure 2 B) contained type A spermatogonia, but almost no type B spermatogonia or spermatocytes, showing that, without AR in Sertoli cells, spermatogonial differentiation remained blocked.

Sertoli Cell Abnormalities in SCARKO-*jsd* Mice

We observed that in the SCARKO-*jsd* mouse testis, the positions of Sertoli cell nuclei were highly abnormal. Unlike wild-type (Figure 1A) and *jsd* mice (Figures 1 C, 2 A, C), in which most of the Sertoli cell nuclei were located in the basal area of tubules and aligned in a circle along the tubule basement membrane, many relatively normal Sertoli cell nuclei were displaced from the basal area and dispersed throughout the tubules in SCARKO-*jsd* mice (Figure 2 B,

D). Clusters of abnormal Sertoli cell nuclei were also observed either near the basement membrane or in the center of the tubule (Figure 2 D).

To obtain a semiquantitative analysis of this phenomenon, we scored tubules as to whether they had fewer than 5 Sertoli nuclei not on basement membrane, more than 5 Sertoli nuclei not on basement membrane, or clusters of Sertoli cell (Figure 3). There were significantly more tubules with ≥ 5 Sertoli cell nuclei detached from the basement membrane and with Sertoli cell clusters in SCARKO-*jsd* mice than in SCARKO mice or in *jsd* mice. While 44% of tubules in *jsd* mice had all Sertoli cells arranged along the basement membrane, only 1% of tubules in SCARKO-*jsd* mice had normal Sertoli cell nuclear localization.

We next examined the morphology of these Sertoli cell nuclei by electron microscopy. There was a slight but consistent increase in the amount of heterochromatin associated with the nuclear membrane of Sertoli cells at the basement membrane in both SCARKO and SCARKO-*jsd* mice (Figure 4 B, D), compared to those found in wild-type and *jsd* mice (Figure 4 A, C). Such an increased level of heterochromatin is typical of immature, prepubertal Sertoli cells (Solari and Fritz, 1978) and Sertoli cells whose maturation is prevented by androgen deprivation (Chemes et al, 1979). Meanwhile, the displaced Sertoli cell nuclei, in both *jsd* and SCARKO-*jsd* mice, were highly abnormal, characterized by more prominent condensed chromatin deposits on nuclear membrane and irregular shapes (Figure 4 E, F). The abnormal chromatin distribution on nuclear membrane was more severe in clustered Sertoli cells than in the scattered Sertoli cells displaced from the basement membrane.

Since the Sertoli cell nuclear position is determined by cytoskeletal attachments between the basal aspect of the cell membrane and nucleus, we examined the intermediate filaments surrounding the nuclei and their connection with hemidesmosomes along the basal aspect of the Sertoli cell membrane as well as the basement membrane structure. Vimentin is the major intermediate filament protein present in structures in mature Sertoli cells that extend from the nucleus to desmosome-like junctions at the surface of the cell (Vogl et al, 1993), and in wild-type mice, it is concentrated around the Sertoli cell nucleus and also in some stalks extending towards the lumen (Supplemental Figure 1 A). The primary localization of vimentin in Sertoli cells of SCARKO, *jsd*, and SCARKO-*jsd* mice (Supplemental Figure 1 B, C, D) was also perinuclear, but the extensions of the vimentin trunks towards the center of the tubule were reduced or absent. The nuclei displaced from the basement membrane and those found in clusters in the central region of the tubules in SCARKO-*jsd* testes also showed the perinuclear of vimentin localization (Supplemental Figure 1 D). By electron microscopy, the intermediate filaments were observed around the nucleus and extending to make contact with hemidesmosome-like junctions along the Sertoli cell membrane in contact with the basement membrane extracellular matrix (Supplemental Figure 2). These structures were comparable among the four genotypes. However in the basement membrane of the seminiferous tubules, which consists of the basal laminae and collagen fibers, the basal laminae in testes of SCARKO and SCARKO-*jsd* mice were consistently thicker than that in testes of wild-type and *jsd* mice.

In order to determine if the ectoplasmic specialization (ES) junctional complexes between Sertoli cells were affected in SCARKO-*jsd* mice, we first examined their ultrastructure in these mice, as well as in wild-type, SCARKO, and *jsd* mice. These complexes are characterized by a pair of parallel Sertoli cell membranes, with both adherens and tight junctions, which are sandwiched between layers of actin bundles and endoplasmic reticulum in wild-type (Supplemental Figure 3 A). These complete structures were also observed in SCARKO, and in *jsd* mice (Supplemental Figures 3 B - E), although in *jsd* mice the tight junctions were not readily found. In SCARKO-*jsd* mice, although some basal junctional complexes consisting of ES and adherens junctions were observed (Supplemental Figure 3 F), they appeared to be less common and/or less well-formed. In addition, we could not find a tight junction in areas we

sampled in these mice. Next we examined the distribution of vinculin, a protein associated with actin filaments at adherens junctions and ES (Vogl et al, 1993). In wild-type, SCARKO, and *jsd* testes, vinculin was observed in the cytoplasm of Sertoli cells and was brightest in the basal region of the tubule (Figure 5 A - C). In SCARKO-*jsd* mice (Figure 5 D), however, the cytoplasmic distribution of vinculin in the Sertoli cells was more uniform, and since there was no lumen formed in the tubules, the staining of vinculin occupied the whole tubule.

Support of Spermatogonial Differentiation in SCARKO-*jsd* Mice

To determine whether failure of spermatogonial differentiation in SCARKO-*jsd* mice was indeed a result of testosterone action on cells other than Sertoli cells or whether Sertoli cell abnormalities were a contributing factor, we treated SCARKO-*jsd* mice with a GnRH-antagonist (acyline) and an AR antagonist (flutamide), since it had previously been shown that GnRH-antagonist treatment alone or with flutamide stimulated spermatogonial differentiation. The seminal vesicle and testis weights were markedly reduced (Table 1), demonstrating that testosterone action was blocked in these mice. As observed in *jsd* mice, treatment with GnRH-antagonist plus flutamide stimulated recovery of spermatogenesis up to the spermatocyte stage in SCARKO-*jsd* mice (Table 2, Figure 6 B). Interestingly, restoration of differentiating germ cells by suppression of testosterone greatly improved the organization of Sertoli cell nuclei in testes of SCARKO-*jsd* mice (Figure 6 D).

Furthermore, treatment with testosterone, given in place of flutamide, reversed the stimulatory effect on spermatogonial differentiation in SCARKO-*jsd* mice because androgen levels were increased and androgen action on other cell types was no longer blocked (Table 2, Figure 6 C). The results clearly demonstrated that testosterone was still the factor maintaining arrest of spermatogonial differentiation in SCARKO-*jsd* mice and further confirmed that AR in Sertoli cells did not mediate this effect.

Discussion

Since AR in Sertoli cells mediates most of the indirect androgen action on germ cells (De Gendt et al, 2004) and specific ablation of Sertoli cell AR in *jsd* mice affected those cells, we initially expected that it would also mediate the testosterone-dependent block in spermatogonial differentiation in *jsd* mice. However, the block of spermatogonial cell differentiation was not reversed in SCARKO-*jsd* mice. Thus, the results of this study conclusively demonstrate that although androgen action on AR in Sertoli cells has significant effects on the structure of these cells, it does not mediate the action of testosterone in blocking spermatogonial differentiation in *jsd* mice.

We considered that the abnormalities in Sertoli cells structure in the absence of androgen might also contribute to their inability to support spermatogonial differentiation in SCARKO-*jsd* mice. Most prominently, the positioning of Sertoli nuclei was drastically disrupted in testes of these mice, compared to previous reports of minimal disorganization and dislocation Sertoli cell nuclei from the basement membrane in SCARKO mice (Tan et al, 2005; Wang et al, 2006). As the SCARKO-*jsd* mice can be a useful model for understanding the requirements for Sertoli cell organization, we further examined Sertoli cell structure in these mice.

The positions of the cell nucleus is believed to be controlled by cytoskeletal elements, which in Sertoli cells appear to be the vimentin intermediate filaments, and their attachment to junctional complexes at the cell membrane (Vogl et al, 1993). Although others have shown that elimination of androgen action on Sertoli cells up-regulated vimentin mRNA levels (Wang et al, 2006) and increased degradation of vimentin protein (Show et al, 2003), our results showed normal localization of vimentin protein and morphologically intact intermediate filament connections between the nuclei and the hemidesmosome junctions in the Sertoli cells

with nuclei near the basement membrane in SCARKO-*jsd* mice. This result suggests that neither disruption of the vimentin filament arrangement nor the loss of anchoring of Sertoli cell nuclei to the basal side of its plasma membrane appear to be the primary reason for the displacement of Sertoli cell nuclei. The only abnormality within the anchoring structure we noted was the increased thickness of basal laminae in the testes of both SCARKO and SCARKO-*jsd* mice, suggesting that the regular structure of the basement membrane does not fully develop when androgenic signaling is disrupted in Sertoli cells, as has also been noted in SCARKO mice (Wang et al, 2006).

Junctions between Sertoli cells and between Sertoli and germ cells can also contribute to maintenance of the Sertoli cell structure. Indeed, in SCARKO-*jsd* mouse testes electron microscopy showed that typical ectoplasmic specialization structures were rare and tight junctions could not readily be found. This is consistent with observations showing that eliminating expression of AR in Sertoli cells increases the permeability of the Sertoli cell barrier and decreases the levels of expression of components of the occluding junctions, claudin 3, claudin 11, and occludin (Meng et al, 2005; Wang et al, 2006).

Consistent with ultrastructural observations, vinculin, one of the component proteins of the ectoplasmic specialization (Vogl et al, 1993) does not show its generally basal localization observed in wild-type, SCARKO, and *jsd* mice and was more uniformly distributed throughout the seminiferous tubule of SCARKO-*jsd* mice. Since vinculin functions in adherens junctions between cells and in focal contacts with the basal lamina, absence of concentrated distribution of vinculin in testes of SCARKO-*jsd* mice may contribute to the sloughing of Sertoli cells from the basal area. The formation of tight junctions and the localized vinculin distribution was, at least, partly dependent on androgen action in the Sertoli cells, although altered vinculin distribution could, in part, be a consequence of the redistribution of the Sertoli cell cytoplasm. Similarly, the localization of the cadherin-catenin complex, another component of adherens junctions, in the Sertoli cell ectoplasmic specialization, was also shown to be dependent on testosterone (Xia et al, 2005).

Although androgenic signaling in Sertoli cells is important to maintain their normal structure and nuclear positioning, presence of differentiated germ cells also appears to be involved. This conclusion is supported here by several lines of evidence. First, even with normal androgen action, *jsd* mice also showed mild disruption and sloughing of Sertoli cells. It should be noted that shrinkage of seminiferous tubules may also be a factor contributing to this abnormal organization. Second, dislocation of the nuclei and sloughing of Sertoli cells in SCARKO mice, which contain some differentiated germ cells, was much milder than that in SCARKO-*jsd* mice. In this case, the absence of androgen-dependent fluid secretion by Sertoli cells in SCARKO-*jsd* mice, which resulted in lack of lumen formation and loss of fluid pressure from the center of tubules, could also contribute to the disrupted organization of Sertoli cells and their nuclei. Third, the role of the differentiated germ cells in maintaining the positioning of the Sertoli cells and their nuclei was also strongly supported by the observation that restoration of germ cells by suppression of testosterone improved Sertoli cell organization in SCARKO-*jsd* mice.

We also are in the process of examining the overall changes in Sertoli cell gene expression in SCARKO-*jsd* by microarray comparison of these testes with *jsd* mice. Preliminary data suggest that the Sertoli cells in these SCARKO-*jsd* mice have equivalent expression of most markers of Sertoli cell maturation as *jsd* mice (Sharpe et al, 2003). Markers of mature Sertoli cells, SGP2 (*Clu*), GATA-1, and p27-Kip1 (*Cdkn1b*) were expressed at equivalent levels. Markers of immature Sertoli cells, AMH and aromatase (*Cyp19a1*), were not expressed. However there was a 4-fold higher expression of cytokeratin 1-18 (*Krt18*), a marker of immature Sertoli cells in the SCARKO-*jsd* mice than in *jsd* mice indicating that Sertoli cell maturation may be somewhat abnormal.

Despite the disrupted organization and some structural and molecular defects, the absence of AR in the Sertoli cells of SCARKO-*jsd* mice cannot be the cause of the inability of spermatogonial differentiation to proceed. These testes are indeed capable of supporting spermatogonial differentiation when the testosterone levels and action were globally blocked. Thus, the target of testosterone producing the spermatogonial block in *jsd* mice must be some other AR-positive somatic cell within or outside the testis. In a related study, we have shown that increased temperature of testis alone is able to stimulate the spermatogonial differentiation in *jsd* mice even more effectively than did suppression of testosterone (Shetty and Weng, 2004). The association between testosterone levels, testicular temperature, and spermatogonial differentiation is currently under further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunohistochemical staining (brown) of androgen receptor in testes of wild-type (A), SCARKO (B), *jsd* (C), and SCARKO-*jsd* (D) mice. The sections were counterstained with hematoxylin (blue). GC, germ cells. SC, Sertoli cells. LC, Leydig cells. PMC, peritubular myoid cells. SMC, smooth muscle cells. Bars, 10 μm.

Figure 2.

Histology of testis in *jsd* (A and C) and *SCARKO-jsd* (B and D) mice. Arrows indicate type A spermatogonia. N, Tubules with normal Sertoli cell nuclear localization. <5, Tubules with less than 5 Sertoli cell nuclei not on basement membrane. ≥ 5 , Tubules with 5 or more Sertoli cell nuclei not on the basement membrane. C, Tubules with a cluster of Sertoli cell nuclei, usually in the center of the tubule. Bars, 20 μm .

Figure 3.

Disruption of Sertoli cell nuclear localization in testes of SCARKO-*Jsd* mice. Localization of Sertoli cell nuclei was evaluated in cross sections of testes in SCARKO, *Jsd*, and SCARKO-*Jsd* mice. A Sertoli cluster is defined as containing more than five nuclei. a and b indicate values for SCARKO-*Jsd* mice that are significantly different from SCARKO and *Jsd*, respectively. Error bars represent MSE.

Figure 4.

Electron micrographs of Sertoli cell nuclei in testes of wild-type (A), SCARKO (B), *jsd* (C and E), and SCARKO-*jsd* (D and F) mice. Thin arrows indicate the Sertoli cell nuclei located in basal area. Arrowheads indicate some abnormal condensed chromatin deposits on the nuclear membrane of Sertoli cells near the basement membrane. Wide arrows indicate individual (filled arrows) or clustered (open arrows) Sertoli nuclei displaced from the basement membrane. The displaced nuclei had varying degree of abnormal condensed chromatin on the nuclear membrane and irregular nuclear outlines with invaginations. Bars, 2 μ m (A, B, C and D) and 10 μ m (E and F).

Figure 5. Localization of vinculin in testes of wild-type (A), SCARKO (B), *jsd* (C), and SCARKO-*jsd* (D) mice. Arrows indicate clusters of Sertoli cells displaced from the BM. Arrowheads indicate some staining of vinculin in the basal region of the tubules. Bar, 20 μ m. All pictures were taken under the same conditions.

Figure 6. Stimulation of recovery of spermatogenesis by suppression of testosterone in SCARKO- *jsd* mice. Hematoxylin-stained testis sections of SCARKO- *jsd* mice without treatment (A), treated with GnRH-antagonist plus flutamide (B, D), or treated with GnRH-antagonist plus testosterone (C). Asterisks indicate the tubules with differentiating germ cells. Sertoli cell arrangement was improved in the tubules in which differentiated germ cells were restored when treated with GnRH-antagonist plus flutamide (D). Bars, 20 μ m.

Table 1

Testis and seminal vesicle weights of wild-type and various transgenic mice and effects of GnRH-antagonist and flutamide or testosterone treatment*

Groups	Mouse genotype (<i>Ar</i> , <i>Amh-Cre</i> , <i>Utp 14b</i>)	Hormone treatment	Testis weight (mg)	Seminal Vesicle weight (mg)
Wild type	<i>Ar^{fllox/Y}</i> , <i>-/-</i> , <i>Utp 14b^{+/+}</i> or <i>Utp14b^{jsd/+}</i>	None	105.9 ± 3.1 ^{a,b}	294 ± 33
SCARKO	<i>Ar^{fllox/Y}</i> , <i>-/-Cre</i> , <i>Utp 14b^{+/+}</i> or <i>Utp14b^{jsd/+}</i>	None	25.9 ± 0.8	307 ± 34
<i>Jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-</i> , <i>Utp 14b^{jsd/jsd}</i>	None	19.5 ± 0.5	302 ± 16
SCARKO- <i>jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-Cre</i> , <i>Utp 14b^{jsd/jsd}</i>	None	17.2 ± 0.6 ^a	286 ± 16
<i>Jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-</i> , <i>Utp14b^{jsd/jsd}</i>	GnRH-antagonist + Flutamide	11.4 ± 0.4 ^c	38 ± 5 ^c
SCARKO- <i>jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-Cre</i> , <i>Utp 14b^{jsd/jsd}</i>		10.8 ± 0.6 ^c	53 ± 7 ^c
<i>Jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-</i> , <i>Utp14b^{jsd/jsd}</i>	GnRH-antagonist + Testosterone	13.3 ± 0.4 ^c	385 ± 42 ^{c,d}
SCARKO- <i>jsd</i>	<i>Ar^{fllox/Y}</i> , <i>-/-Cre</i> , <i>Utp 14b^{jsd/jsd}</i>		13.2 ± 0.5 ^c	399 ± 24 ^{c,d}

* There were between 5 and 12 mice in each group

^a Untreated mice were significantly different from untreated SCARKO mice

^b Untreated mice were significantly different from untreated *jsd* mice

^c Treated mice were significantly different from untreated mice of same genotype

^d Mice treated with GnRH-antagonist + testosterone were significantly different from mice of same genotype treated with GnRH-antagonist + flutamide

Table 2

Spermatogonial differentiation in testes of *jsd* and SCARKO-*jsd* mice as measured by the tubule differentiation index* (TDI) and the effects of hormone treatment on the TDI

	No treatment	GnRH-antagonist + Flutamide	GnRH-antagonist + Testosterone
<i>Jsdl</i>	6.3 ± 4.8	34.1 ± 9.8 ^a	4.3 ± 0.6 ^b
SCARKO- <i>jsd</i>	7.0 ± 3.3	55.3 ± 7.7 ^a	5.9 ± 2.1 ^b

* The TDI is defined as the percentage of tubules that contain three or more differentiating germ cells at the B spermatogonial stage or beyond.

^a Treated mice were significantly different from untreated mice of same genotype

^b Mice treated with GnRH-antagonist + testosterone were significantly different from mice of same genotype treated with GnRH-antagonist + Flutamide